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Cytoskeleton remodeling is crucial in many cellular events, including cell adhesion, spreading and motility. The Rho family of small GTPases (Rho, Rac and Cdc42) are signal transducers that regulate cytoskeletal dynamics. However, little is known about the mechanisms by which Rho GTPases induce cytoskeletal changes. Complex systems of actin depolymerization/polymerization, actin-myosin interactions and coupling of actin binding proteins with actin filaments regulate cytoskeleton remodeling. p-21 activated kinase (PAK), an effector molecule activated by Rac and Cdc42, has been previously implicated in cytoskeletal changes. Here we describe two novel substrates of PAK, which are important regulators of the cytoskeleton. Myosin light chain kinase (MLCK), which mediates phosphorylation of the regulatory myosin light chain and thus controls contractility and tension of the cytoskeleton, is phosphorylated by PAK both in vitro and in vivo. This phosphorylation results in decreased activity of MLCK and, hence, decreased contractility. Lim-kinase phosphorylates and inactivates the small actin binding protein cofilin/actin depolymerizing factor (ADF). PAK phosphorytates Lim-kinase and increases Lim-kinase-mediated phosphorylation of cofilin 10 fold, thereby promoting actin polymerization. Consequently, one mechanism by which activated GTPases may regulate cytoskeletal remodeling is through PAK-mediated signaling events.

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Suzanne Sanders 8-31-99

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Introduction

Tumor invasion and metastasis are the major cause of cancer mortality. This is particularly true for breast cancer since metastasis of the primary tumor leads to tumor inaccessibility and, consequently, greater mortality. Many studies have shown the importance of signaling molecules in changes that are associated with transformed phenotypes (1-3). In fact, many signaling molecules are affiliated with cytoskeletal changes that accompany the motile or metastatic phenotype. The Rho family of small GTPases (Rho, Rac and Cdc42) are members of the Ras superfamily; these regulate cell function via conversion between a GTP-bound active state and a GDP-bound inactive form. Recently, it has become clear that the Rho family mediates morphological and cytoskeletal changes of both normal and transformed cells (4,5). Rho activation leads to stress fiber formation and focal adhesions. Activation of Rac leads to membrane ruffles and lamellipodia formation. Similarly, Cdc42 regulates the extension of actin filament bundles into filopodia. The molecular mechanisms by which the Rho family of GTPases regulates cytoskeleton remodeling is not well understood.

Dynamic rearrangement of the cytoskeleton is, in part, driven by actin polymerization/depolymeriation and actin-myosin II interactions. Myosins II (which are found in all cells and are constructed of two heavy chains and four light chains) are mechanoenzymes which generate force along actin filaments and, thus, are crucial for cell movements, including cytokinesis, pseudopod formation, polarized growth and cell migration (6-8). Changes in the expression of myosin isoforms have been linked to the transformed phenotype in both melanoma and breast cancers (9-11). Recently, Rho GTPase has been shown to regulate myosin activity though Rho kinase, an effector molecule for Rho (12,13). Rho kinase phosphorylates myosin phosphatase, which removes phosphate groups from myosin light chain, and inhibits its function. Consequently, one mechanism by which Rho effects cytoskeletal dynamics is by increasing myosin light chain phosphorylation through the inhibition of dephosohorylation.

In this grant we proposed to examine the effects p21-activated kinase (PAK), an effector molecule for Rac and Cdc42, had on cytoskeleton dynamics and how this relates to the phenotype and behavior of breast cancer cells.

Results

Myosin light chain kinase (MLCK) increases in phosphorylation at Ser-19 on myosin light chain (MLC) are essential for force generation by myosin II. During postmitotic cell spreading, phosphorylation of this site is elevated when compared to completely spread cells (14). The spreading edge of a cell is analogous to the leading edge of a migrating cell and collectively these are referred to as moving edges. Furthermore, cell spreading is a prerequisite for cell migration. Aim 2 of this grant was to evaluate the effect PAK may have on myosin II phosphorylation. Therefore, we looked at the effects PAK had on MLC phosphorylation during cell spreading. When BHK-21 cells were transfected with PAK1 T423E (catalytically active) and placed in an adhesion assay, spreading was largely inhibited. However, cells expressing Lac Z or PAK WT spread normally (15). Inhibitors of myosin or MLCK showed similar results to PAK T423E expressing cells. Cells overexpressing PAK T423E, PAK WT, Lac Z, or control cells (non-transfected) were allowed to attach and spread on a fibronectin matrix, then lysed at various time points. Immunoblot analysis was performed using an antibody that recognizes the Ser-19 phosphorylated form of MLC (18). During cell spreading the control cells show a gradual increase in MLC phosphorylation, with the maximum at the 45-min time point. However, PAK1 T423E expressing cells show little to no phosphorylation of MLC at any of the time points. Cells transfected with Lac Z or PAK WT showed a level of MLC phosphorylation similar to control cells. These data suggest that in vivo, catalytically active PAK1 acts to inhibit phosphorylation of MLC on Ser-19 (15).

The calcium-calmodulin dependent myosin light chain kinase (MLCK) phosphorylates MLC on Ser-19 and is known in vivo to be responsible for promoting the force generating ability of myosin II. Therefore, in order to understand PAK's role in decreasing phosphorylation of MLC, we looked at its effect on MLCK. In vitro phosphorylation experiments demonstrate that PAK 1 can phosphorylate MLCK and this phosphorylation is independent of calmodulin. PAK 1 phosphorylation of MLCK lead to a 50% reduction in the catalytic activity of MLCK (15). This data suggests that catalytically active PAK inhibits MLC phosphorylation by phosphorylating MLCK and downregulating its activity.

To test the ability of PAK to inactivate MLCK in vivo, cells were transfected with PAK1 WT and T423E, lysed, and MLCK was immunopercipitated and assayed for activity. MLCK immunopercipitated from PAK T423E cells showed a significant decrease in activity when compared to MLCK from control or PAK WT expressing cells (15). This data confirms our in vitro data that PAK phosphorylation of MLCK inhibits its activity.

We have also found that PAK phosphorylates and activates another enzyme important in controlling cytoskeleton dynamics, Lim-kinase (16). Lim-kinase phosphorylates and inactivates the small actin binding protein cofilin/actin depolymerizing factor (ADF). Recent studies have indicated Lim-kinase acts downstream of Rac (17). PAK phosphorylated Lim-kinase at threonine 508 and increased Lim-kinase-mediated phosphorylation of cofilin ~10 fold in vitro. In vivo, activated Rac increased association

of PAK with Lim-kinase, which required structural determinants in both the NH_2-terminal regulatory and COOH-terminal catalytic domains of PAK. These data suggest a model by which the activation of PAK by Rac/Cdc42 leads to enhanced binding of Lim-kinase and effective phosphorylation by PAK. A catalytically inactive Lim-kinase interfered with Rac-, Cdc42-and PAK-dependent cytoskeletal changes. A PAK-specific inhibitor, corresponding to the PAK autoinhibitory domain, blocked Lim-kinase-induced cytoskeletal changes.

Aim 3 of this grant was to examine the functional importance of PAK on breast cancer cell migration. Detailed cellular analysis of breast cancer cell lines has been hindered by the low protein expression and number of transfected cells that microinjection and other traditional methods render. Therefore, we perfected a viral gene expression system to overcome this problem. This system has allowed us to transiently express Rho family GTPases and PAKs with an efficiency of greater than 95% in many breast cancer cell lines. We are now able to study a population of cells that can be placed into bioassays, including migration or adhesion assays. Using the virus system to transfect cells, our preliminary results indicate that high levels of activated PAK decreases cell migration. This would be consistent with PAK's ability to decrease MLCK activity and increase Lim-kinase activity.

Recently, in collaboration with the Knaus lab here at Scripps, we have shown that some breast cancer cell lines have constitutively activated PAK (18). Most of the cell lines showing increased PAK activity also had constitutively active Rho GTPase. However, one of these cell lines, ZR-75, had activated PAK with no activated GTPase. Interestingly, these cells has many of the previously reported characteristics of cells transfected with constitutively activated PAK's (Figure 1). For example, overexpression of PAK T423E has been shown to desolve stress fibers (19); note the lack of stress fibers in the ZR-75 cells (Figure 1a). Activated PAK's have also been reported to cause dorsal ruffling (20). ZR-75 cells contain many large dorsal ruffles (Figure 1b). ZR-75 cells spread very slowly, taking 10 hrs to completely spread, where control cells (BHK-21 and MDA-231) are completely spread in 2 hrs (Figure 2). This is reminiscent of BHK-21 cells overexpressing PAK T423E, which show inhibition of spreading (15). When ZR-75 cells are placed in a migration assay they migrate much slower than control cells (MDA-231 breast cancer cells with no activated PAK) (Figure 3).

Conclusion

Cell adhesion, migration and invasion plays critical roles in the pathogenesis of tumor metastasis. Comprehending cytoskeleton dynamics is pivotal in understanding the complexities of metastasis. Thus, our studies have first focused on cell spreading in order to understand the complexities of the cytoskeleton at the moving edge of the cell. Our lab and others have shown that PAK localizes to Rac-induced membrane ruffles (20) and mutationally active forms can cause cytoskeleton changes (19, 20, 21). These data suggest a role for PAK in cytoskeletal remodeling. Data presented in this report further demonstrate the importance of PAK and suggest a possible mechanism by which it influences the cytoskeleton. We describe two novel targets for PAK, MLCK and Lim-

kinase. Both of these kinases are important regulators of cytoskeleton dynamics. Aim 2 in our grant proposal was to determine if PAK affected myosin phosphorylation (Technical Objective 2: task 3 and 4). We believe that these results satisfy the objective of Aim 2.

At present we are working on Aim 3 in our proposal, which is to examine the importance of PAK-myosin interactions on breast cancer cell migration. With the development of the viral gene expression system, we are now able to transfect breast cancer cells with high enough transfection efficiency to do migration assays in Boyden chambers (Technical Objective 3: task 5 and 6). These experiments are currently ongoing.

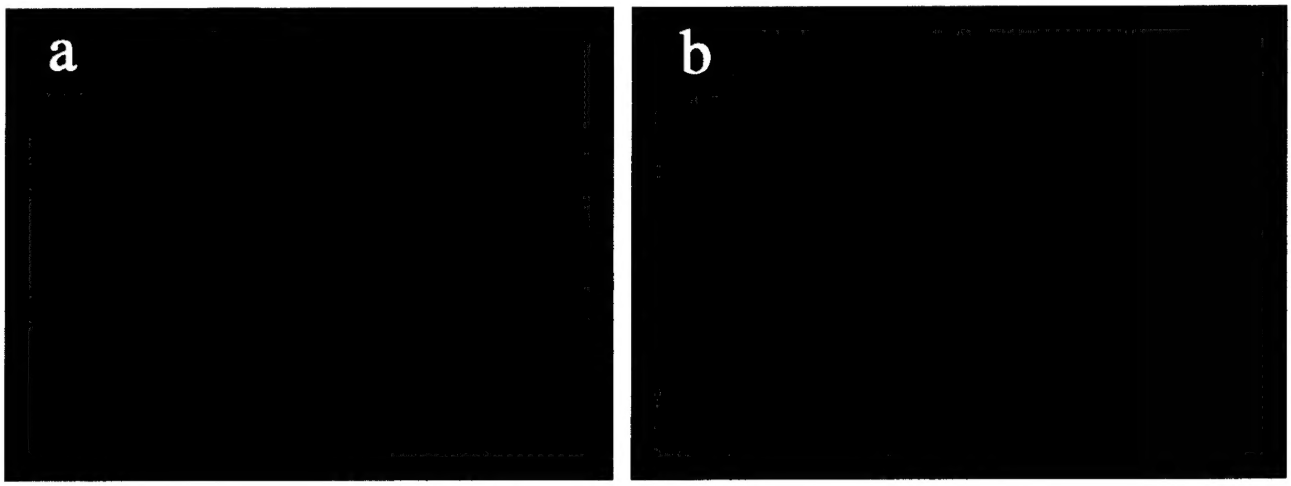

Figure 1: ZR-75 cells grown on coverslips, fixed and stained with phalloidin to show filamentous actin. A; Note the lack of stress fibers. B; Many of these cells have dorsal ruffles. Both morphologies are common in cells overexpressing activated PAK's.

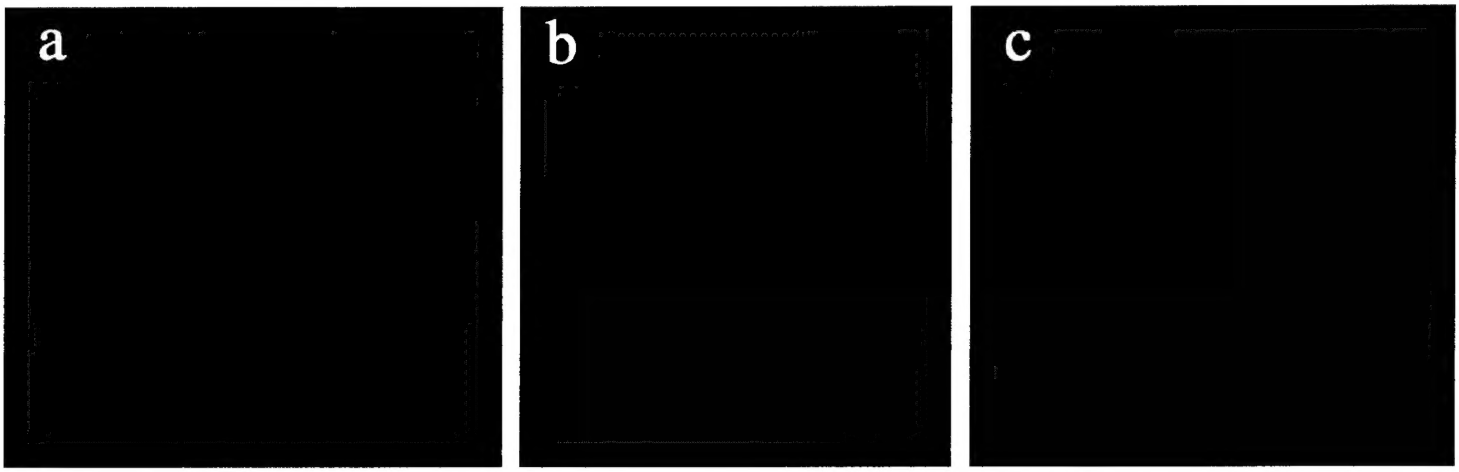

Figure 2: Adhesion assay; cells were harvested, plated on cover slips coated with fibronectin, and allowed to spread for 2 hrs, then fixed and stained with phalloidin. A; BHK-21 cells (fibroblast) are completely spread. B; MDA-231 cells (breast cancer cell line with no activated PAK) were also fully spread within 2 hrs. C; ZR-75 cells (breast cancer cell line with constitutively active Pak) were still rounded after 2 hrs, similary to reported data for BHK-21 cells overexpressing PAK T423E (15).

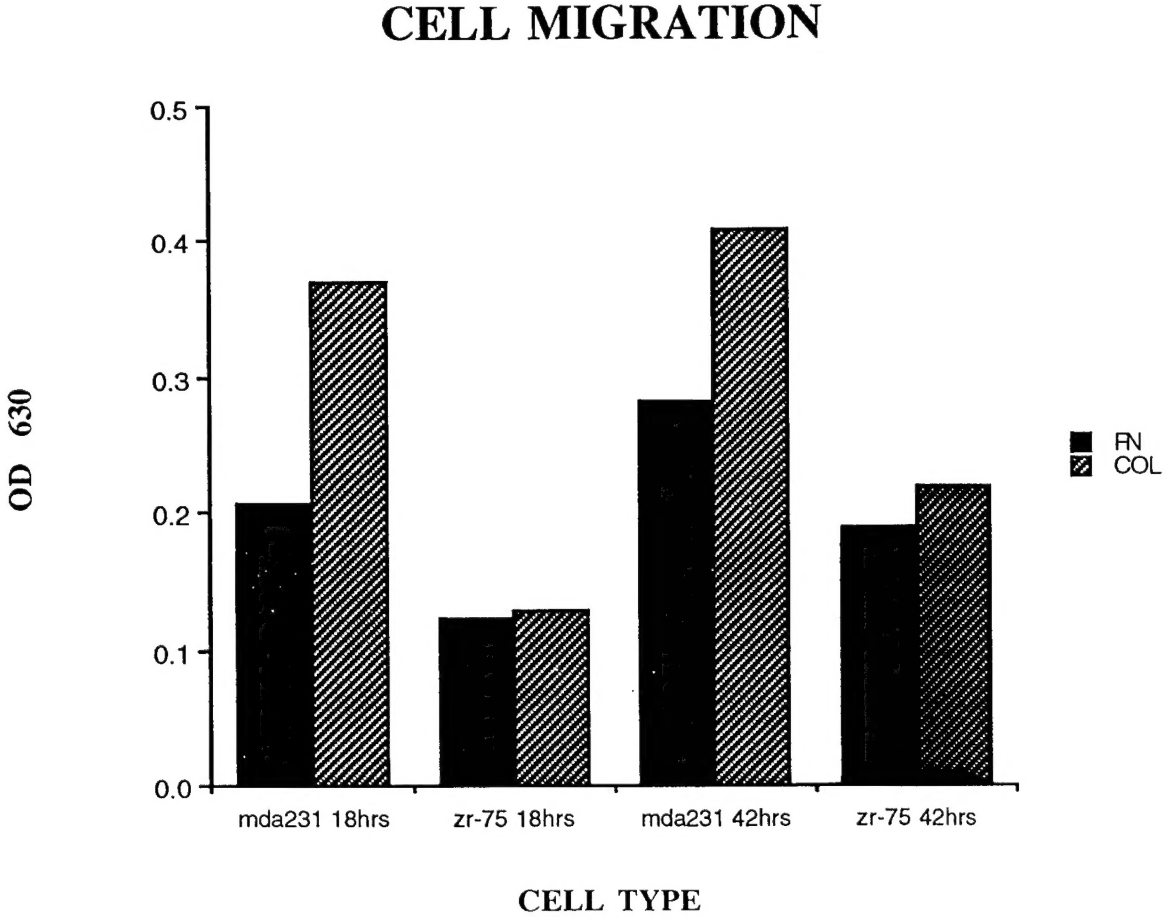

Figure 3: Cell migration assay; ZR-75 cells and MDA-231 cells were allowed to migrated for 18 or 42 hrs on membranes coated with collagen or fibronectin. ZR-75 cells, which have constitutively active PAK, migrate much slower than control MDA-231 cells (no activated PAK). The decrease migration of ZR-75 cells was independent of time point and matrix protein.

References

1. Qui, R-G., J. Chen, D. Kirn, F. McCormick and M. Symons (1995) An essential role for rac and ras transformation. *Nature*, 374:457-459

2. Prendergast, G.C., R. Khosravi-Far, P.A. Solski, H. Kurzowa, P.F. Lebowitz and C.J. Der (1995) Critical role of rho in cell transformation by oncogenic ras *Oncogene*, 10:2289-2296

3. Renshaw, M.W., E. Lea-Chou, G.M. Bokoch and J.P. Wang (1995) Rac is required for v-abl tyrosine kinase to activate mitogenesis. *Nature*, submitted

4. Ridley, A.J., H. F. Paterson, C.L. Johnston, D. Diekmann and A. Hall (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, 70:401-410

5. Hall, A. (1994) Small GTP-binding proteins and the regulation of the actin cytoskeleton. Ann. Rev. *Cell Biol.*, 10: 31-54

6. Wolenski, J.S. (1995) Regulation of calmodulin-binding myosins. *Trends Cell Bio* 5:310- 316.

7. de Lanerolle, P. and Paul, R.J. (1991) Myosin phosphorylation/dephosphorylation and regulation of airway smooth muscle contractility. *Am. J. Physiol.*, 261:L1-L14.

8. Wilson, A.K., Pollenz, R.S., Chisholm, R.L. and de Lanerolle, P. (1992) The role of myosin I and II in cell motility. *Cancer and Metastasis Reviews*, 11:79-91.

9. Kelley, C.A. and R.S. Adelstein (1990) The 204-kDa smooth muscle myosin heavy chain is phosphorylated in intact cells by casein kinase II on a serine near the carboxyl terminus. *J. Bio. Chem.*, 265:17876-17882

10. Fink-Puches, R., and J. Smolle (1993) Cytoskeleton and motility: An immunohistological and computer simulation analysis of melanocytic skin tumors. *J. Cutan. Path.*, 20:130-136

11. Maupin, P., C.L. Phillips, R.S. Adelstein and T.D. Pollard (1994) Differential localization of myosin-II isozymes in human cultured cells and blood cells. *J. Cell Sci.* 107:3077-3090

12. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Kakano, T., Matsuura, Y. and Kaibuchi, K. (1996) Phosphorylation and activation of myosin by rho-associated kinase (rho kinase). *J. Biol. Chem.*, 271: 20,246-20,249.

13. Kimura, K, Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) Regulation of myosin phosphatase by rho and rho-associated kinase. *Science* 273: 245-248.

14. Yamakita, Y., Yamashiro, S., and Mataumura, F. (1994) In vivo phosphorylation of regulatory light chain of myosinII suring mitosis of cultured cells. *J. Cell Biol.* 124:129-137.

15. Sanders, L.C., Matsumura, F., Bokoch, G.M., and de Lanerolle, P. (1999) Inhibition of myosin light chain kinase by p21-activated kinase. *Science* 283:2083-2085.

16. Edwards, D.C., Sanders, L.C., Bokoch, G.M. and Gill, G.N. (1999) Activation of LIM-kinase by PAK couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nature Cell Bio.* 1:253-259.

17. Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O. and Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 393:805-809.

18. Mira, J-P., Benard, V., Groffen, J., Sanders, L.C. and Knaus, U.G. (1999) Constitutive activation of Rac3 mediates proliferation in breast cancer cells by p21-activated kinase-dependent pathways. submitted

19. Manser, E., Huang, H.-Y., Loo, T.-H., Chen, X.-Q., Dong, J.-M., Leung, T. and Lim, L. (1997) Expression of constititively active α-PAK reveal effects of the kinase on actin and focal complexes. *Mol. Cell. Biol.* 17:1129-1143.

20. Dharmawardhane, S., Sanders, L.C., Martin, S.S., Daniels, R.H. and Bokoch, G.M. (1997) Localization of p21 activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. *J. Cell Biol.* 138:1265-1278.

21. Sells, M.A., Knaus, U.G., Ambrose, D., Bagrodia, S., Bokoch, G.M., and Chernoff, J. (1997) Human p21 activated kinase regulates actin reorganization in mammalian cells. *Curr. Biol.* 7:202-210

Appendices:

- PAK activity is important for cell spreading.
- Myosin activity is important for cell spreading.
- PAK activity decreases MLC phosphorylation, in vivo.
- PAK phosphorylates and inactivates MLCK.
- PAK phosphorylates and activates LIM-Kinase.

Publications

Sanders, L.C., Matsumura, F., Bokoch, G.M., and de Lanerolle, P. (1999) Inhibition of Myosin Light Chain Kinase by p21-Activated Kinase. *Science* 283:2083-2085.

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Presentations

1999 Keynote Address, University of Calif., Riverside - Graduate Student Retreat

Inhibition of Myosin Light Chain Kinase by p21-Activated Kinase

Luraynne C. Sanders,[1] Fumio Matsumura,[2] Gary M. Bokoch,[1]* Primal de Lanerolle[3]*

p21-activated kinases (PAKs) are implicated in the cytoskeletal changes induced by the Rho family of guanosine triphosphatases. Cytoskeletal dynamics are primarily modulated by interactions of actin and myosin II that are regulated by myosin light chain kinase (MLCK)–mediated phosphorylation of the regulatory myosin light chain (MLC). p21-activated kinase 1 (PAK1) phosphorylates MLCK, resulting in decreased MLCK activity. MLCK activity and MLC phosphorylation were decreased, and cell spreading was inhibited in baby hamster kidney–21 and HeLa cells expressing constitutively active PAK1. These data indicate that MLCK is a target for PAKs and that PAKs may regulate cytoskeletal dynamics by decreasing MLCK activity and MLC phosphorylation.

Cytoskeletal remodeling is important in many cellular responses, including cell adhesion, spreading, and motility (*1–3*). Rho family members of small guanosine triphosphatases (GTPases)—Rho, Rac, and Cdc42—have been implicated as critical regulators of cytoskeletal changes (*4, 5*). PAK1, an effector molecule activated by Rac and Cdc42, localizes to Rac-induced membrane ruffles (*6*), and mutationally activated PAKs induce cytoskeletal rearrangement (*7, 8*), suggesting a role for PAKs in cytoskeletal dynamics. Cytoskeletal dynamics are primarily modulated by actin and myosin II (*9–11*). Because actin–myosin II interactions are regulated by the phosphorylation of the regulatory MLC by MLCK (*12, 13*), we examined the effects of PAK1 on MLCK activity.

Phosphorylation of Ser^{19} of the regulatory MLC stimulates the actin-activated adenosine triphosphatase (ATPase) activity of myosin II and regulates the force-generating ability of myosin II in vivo (*1, 10, 12, 13*). Although MLCK requires calcium and calmodulin for activity (*12*), phosphorylation by other protein kinases can increase (*14*) or decrease (*15, 16*) MLCK activity. Therefore, we investigated the possibility that PAK1 phosphorylates and regulates MLCK activity. PAK1 phosphorylated MLCK in vitro and caused the incorporation of ~1 mol PO_4/mol MLCK (Fig. 1B). This phosphorylation was independent of calmodulin binding to MLCK (*17*). Under conditions in which we obtained stoichiometric phosphorylation of the enzyme by PAK1, the catalytic activity of MLCK was decreased by ~50% when assayed at a saturating calmodulin concentration (Fig. 1) (*18*). This inhibition appears to be a direct effect on the maximum velocity (V_{max}) and not an effect on the affinity of MLCK for calmodulin ($K_{calmodulin}$) (*17*). This is in contrast to MLCK phosphorylation by cyclic adenosine 5′-monophosphate–dependent protein kinase and calmodulin-dependent kinase II, which change the $K_{calmodulin}$ without affecting the V_{max} (*15, 16*). Thus, the cellular effects of PAK could be mediated through the phosphorylation and inactivation of MLCK and a decrease in MLC phosphorylation.

We studied cell adhesion and cell spreading, which result in the activation of PAK (*19*). Baby hamster kidney–21 (BHK-21) cells were transfected with wild-type PAK1 (wtPAK1) or with a constitutively active PAK1 [$Thr^{423} \rightarrow Glu^{423}$ (T423E)] (*20*), and equal expression of wtPAK1 or PAK1 (T423E) was confirmed by protein immunoblot analysis. Control cells and those expressing PAK1 (T423E) were placed on fibronectin-coated coverslips and allowed to adhere and spread for 2 hours (*21*). Cells were then fixed and examined with fluorescence microscopy. Nontransfected control cells, virus controls encoding LacZ, and cells expressing wtPAK1 attached and spread normally, with 80% of the cells (n = 271) showing a well-spread morphology at 2 hours (Fig. 2A). Phalloidin staining revealed a typical fibroblastlike morphology with numerous stress fibers (Fig. 2A). Cells expressing PAK1 (T423E) attached to fibronectin normally, but cell spreading was reduced, and only 27% of the cells (n = 303) had spread in 2 hours (Fig. 2A).

Cell adhesion and cell spreading on the extracellular matrix require active cytoskeletal remodeling that is dependent on actin polymerization and the interaction of actin with myosin II (*2, 10, 22*). Actin–myosin II interactions are regulated by the phosphorylation of MLC (*13, 14*), and MLC phosphorylation on Ser^{19} increases during postmitotic cell spreading at the leading edge and returns to the baseline level in completely spread cells (*22, 23*). Furthermore, 2,3-butanedione monoxime (BDM) reversibly inhibits the ATPase activity of nonmuscle myosin II and the spreading of postmitotic cells (*22*). We confirmed the participation of myosin II in spreading by showing that BDM (20 mM) inhibits the spreading of BHK-21 cells for up to 90 min (Fig. 2B). The inhibition of cell spreading by BDM was dose-dependent over the concentration range of 2 to 50 mM (*24*). It was also reversible. The cells had spread normally 45 min after the BDM was removed (Fig. 2B). Similarly, cell spreading was inhibited by the MLCK inhibitor ML-7 (*25*), indicating that spreading requires MLCK activity (*24*).

We also analyzed the effect of PAK1 expression on MLCK activity and the phosphorylation of MLC in vivo. Control cells or those cells that expressed PAK1 (T423E) were lysed, and endogenous MLCK was immunoprecipitated and assayed for activity in the presence of a saturating concentration of calmodulin (*18*). MLCK that was immunoprecipitated from cells expressing PAK1 (T423E) showed decreased activity in comparison to that of MLCK immunoprecipitated from control cells or from cells expressing wtPAK1 (Fig. 3).

Immunoblot analysis with an antibody that specifically recognizes MLC phosphorylated on Ser^{19} (*26*) showed a gradual increase in phosphorylation during the spreading of nontransfected control cells (Fig. 4). Cells that were transfected with control plasmids (LacZ or wtPAK1) exhibited similar increases in phospho-

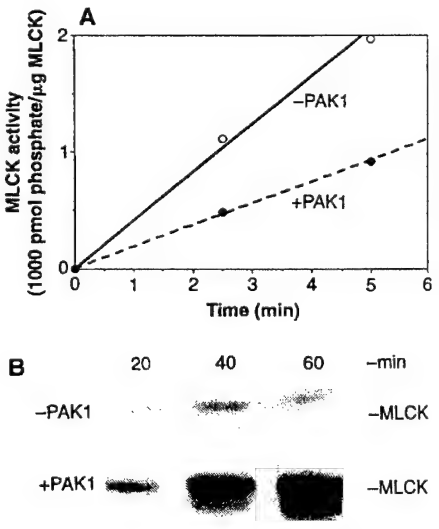

Fig. 1. (**A**) Activity of MLCK incubated with (solid circles) or without (open circles) GST PAK1. MLCK activity was determined with purified MLCs as substrate in the presence of a saturating concentration of calmodulin (*18*). The solid line shows control MLCK activity; the dashed line shows MLCK activity after PAK1 phosphorylation. (**B**) An autoradiogram of MLCK incubated alone (–PAK1) or with PAK1 (+PAK1) for 20, 40, or 60 min and then subjected to SDS-PAGE and autoradiography.

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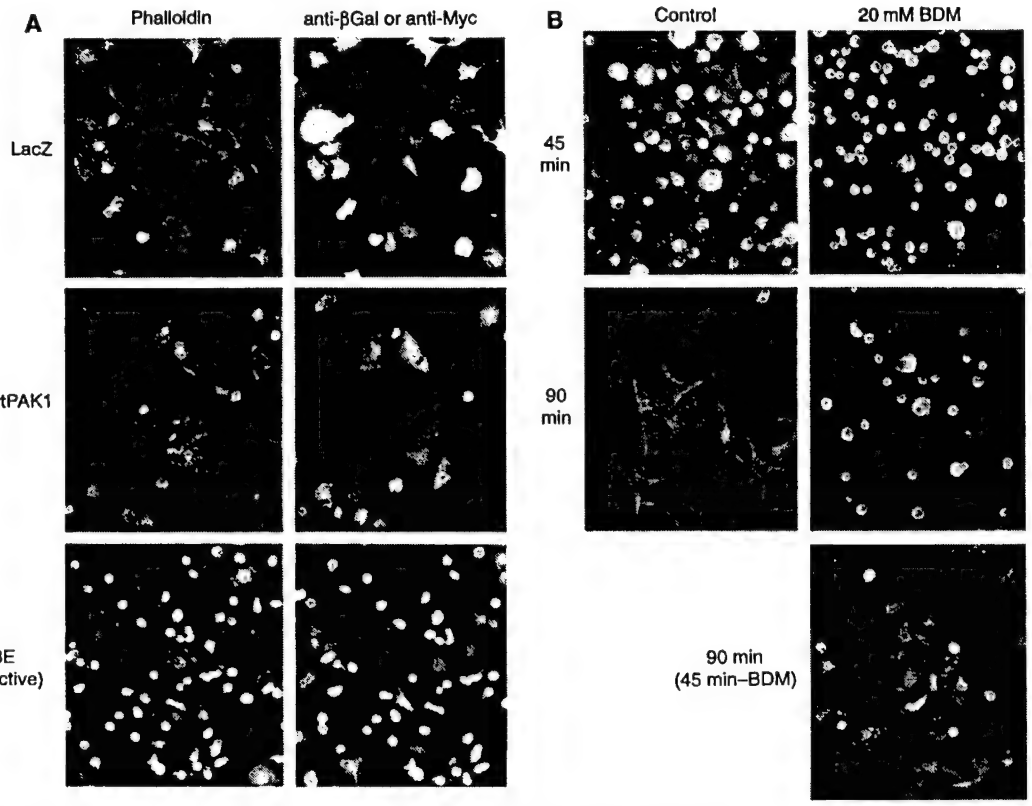

Fig. 2. Inhibition of cell spreading after expression of PAK1 (T423E) or after the addition of inhibitors of myosin ATPase activity. (**A**) Cells were transfected with LacZ, wtPAK1, or PAK1 (T423E) (*20*), allowed to express protein for 6 hours, harvested, and placed on coverslips that were coated with fibronectin (*21*). After 2 hours, the cells were fixed and stained with phalloidin (left panels), with an antibody to β-Gal (top right), or with an antibody to Myc (middle and bottom right panels) to visualize cells expressing wtPAK1 or PAK1 (T423E). (**B**) BHK-21 cells were placed on fibronectin-coated coverslips either in the presence or in the absence of 20 mM BDM (*21*). Cells were fixed after 45 or 90 min and stained with phalloidin. The bottom panel shows cells in which the BDM was washed away after 45 min and incubated for another 45 min.

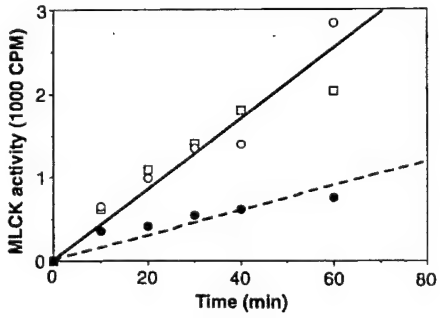

Fig. 3. Decreased MLCK activity in cells expressing PAK1 (T423E). MLCK was immunoprecipitated from nontransfected HeLa cells (open squares) or from cells transfected with wtPAK1 (open circles) or PAK1 (T423E) (solid circles). The immunoprecipitates were then assayed for MLCK activity with MLC as substrate (*18*). Solid line, MLCK activity in control cells; dashed line, MLCK activity in cells expressing PAK (T423E).

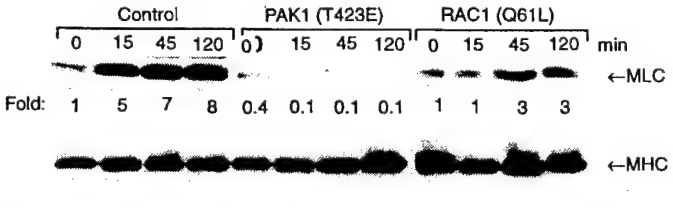

Fig. 4. Inhibition of MLC phosphorylation in BHK-21 cells. Control cells, cells expressing PAK1 (T423E), or cells expressing Rac1 (Q61L) were placed in fibronectin-coated six-well plates. At the indicated time points, cells were lysed, and MLC that was phosphorylated on Ser^{19} was detected by immunoblotting (*26*). The numbers at the bottom of each lane give the increase over the control for each data point by phosphoimager quantification. The bottom row shows that equivalent amounts of myosin were present in each sample, as determined by immunoblotting with an antibody to MHC.

rylation of MLC. In contrast, cells expressing PAK1 (T423E) showed reduced phosphorylation at all time points (Fig. 4). Consistent with the ability of activated PAK1 to decrease MLC phosphorylation, MLC phosphorylation is decreased in cells expressing Rac1 [Gln^{61} → Leu^{61} (Q61L)], the upstream activator of PAK (Fig. 4). This inhibitory effect of Rac1 (Q61L) could be partially reversed by coexpressing a PAK1 autoinhibitory domain (amino acids 83 through 149) (*24*). Thus, catalytically active PAK1 may act in vivo to inhibit MLCK activity, MLC phosphorylation on Ser^{19}, and cell spreading.

The dynamic nature of cell rearrangement and motility requires complex coordinated regulation of the cytoskeleton by Rho, Rac, and Cdc42 (*3–5, 27*). Rho kinase, the Rho effector molecule, phosphorylates myosin phosphatase and inhibits its activity (*28*) and may directly phosphorylate MLC as well (*29*). Both activities serve to increase the phosphorylation of MLC and to stimulate contractility (*28, 29*). This regulatory activity of Rho may be important in the contractile events necessary for maintaining the rigidity that is characteristic of fully spread, stationary fibroblasts (*4, 27–29*).

In contrast, Rac and Cdc42 appear to regulate actin rearrangements that are important in the early stages of cell spreading (*4*) as well as dynamic morphological changes that are associated with cell migration (*9, 10*). Our results identify MLCK, which regulates myosin II function, as a specific target for PAK. The data of Fig. 3 indicate that a decrease in MLCK activity of 50 to 60%, induced by expression of PAK1 (T423E), is sufficient to inhibit MLC phosphorylation by 80 to 90%. A change in MLCK activity of this magnitude is likely to be sufficient to account for the observed decrease in MLC phosphorylation. However, we cannot rule out the possibility that PAK also modulates other pathways that affect the steady-state level of MLC phosphorylation, although preliminary

data indicate that PAK does not stimulate MLC phosphatase activity (*30*). Thus, regulation of MLCK activity and, hence, MLC phosphorylation appears to be an important component of Rac- and Cdc42-dependent cytoskeletal remodeling in spreading cells. Because Rho kinase and PAK have opposing effects on MLC phosphorylation, the integrated cellular response to the activation of Rho, Rac, and Cdc42 may depend on the timing of GTPase activation as well as the intracellular localization and extent of MLC phosphorylation.

References and Notes

1. A. K. Wilson *et al.*, *Cancer Metastasis Rev.* **11**, 79 (1992).
2. T. J. Mitchison and L. P. Cramer, *Cell* **84**, 371 (1996).
3. K. Burridge and M. Chrzanowska-Wodnicka, *Annu. Rev. Cell Dev. Biol.* **12**, 463 (1996).
4. C. D. Nobes and A. Hall, *Cell* **81**, 53 (1995).
5. A. Hall, *Science* **279**, 509 (1998).
6. S. Dharmawardhane *et al.*, *J. Cell Biol.* **138**, 1265 (1997).
7. M. A. Sells *et al.*, *Curr. Biol.* **7**, 202 (1997).
8. R. H. Daniels, P. S. Hall, G. M. Bokoch, *EMBO J.* **17**, 754 (1998).
9. R. L. Debiasio *et al.*, *J. Cell Biol.* **107**, 2631 (1988).
10. A. K. Wilson *et al.*, *ibid.* **114**, 277 (1991).
11. J. Howard, *Nature* **389**, 561 (1997).
12. P. de Lanerolle and R. J. Paul, *Am. J. Physiol.* **261**, L1 (1991).
13. A. P. Somlyo and A. V. Somlyo, *Nature* **372**, 231 (1994).
14. R. L. Klemke *et al.*, *J. Cell Biol.* **137**, 481 (1997).
15. P. de Lanerolle, M. Nishikawa, D. A. Yost, R. S. Adelstein, *Science* **223**, 1415 (1984).
16. M. G. Tansey *et al.*, *J. Biol. Chem.* **267**, 12511 (1992).
17. P. de Lanerolle *et al.*, in preparation.
18. Purified smooth muscle MLCK (5.2 μg) was incubated at 30°C for 1 hour by itself (autophosphorylation control) or with constitutively active recombinant glutathione S-transferase–PAK1 (0.5 μg) [U. G. Knaus, S. Morris, H.-J. Dong, J. Chernoff, G. M. Bokoch, *Science* **269**, 221 (1995)] at 30°C in buffer containing 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.1 mM ^{32}P-labeled adenosine 5′-triphosphate (specific activity ~2000 cpm/pmol), and 20 mM tris-HCl (pH 7.5). Aliquots were removed at various times and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. To measure MLCK activity, we incubated autophosphorylated or PAK1-phosphorylated MLCK (50 ng) at room temperature as described above, with 0.5 mM $CaCl_2$, 10^{-7} M calmodulin, 5 mM DTT, and purified regulatory MLC (10 μg). Aliquots were removed at various times and subjected to SDS-PAGE. The bands representing MLC were excised and counted. To quantify changes in MLCK activity in cells, we immunoprecipitated MLCK from nontransfected HeLa cells or from HeLa cells expressing LacZ, wtPAK1, or PAK1 (T423E) as described [P. de Lanerolle, G. Gorgas, X. Li, K. Schluns, *J. Biol. Chem.* **268**, 16883 (1993)] with affinity-purified goat antibodies to MLCK [P. de Lanerolle *et al.*, *Circ. Res.* **68**, 457 (1991)] and protein A–Sepharose. The beads were washed extensively and then assayed for MLCK activity as described. HeLa cells were used in these experiments because the MLCK antibody reacts poorly with BHK-21 MLCK on protein immunoblot analysis.
19. L. S. Price, J. Leng, M. A. Schwartz, G. M. Bokoch, *Mol. Biol. Cell* **9**, 1863 (1998).
20. The cDNAs encoding wtPAK1 or a constitutively active form of PAK1 in which Thr^{423} is mutated into Glu (T423E) were expressed in cells with the Semliki Forest virus Gene Expression System (Life Technologies, Gaithersburg, MD). The cDNAs were amplified using the polymerase chain reaction with primers that contained a Bam HI restriction enzyme site and a Myc tag at the 5′ end. These constructs were subcloned into the Bam HI site of the Semliki Forest vector pSFV3. In vitro transcription of linearized pSFV3 constructs and pSFV-Helper2 was done with SP6 RNA polymerase. RNA transfection of BHK-21 cells was performed by electroporation as described [K. Lundström *et al.*, *Eur. J. Biochem.* **224**, 917 (1994)], yielding recombinant viral stocks of ~10^7 plaque-forming units per milliliter. Viral stocks were stored at −80°C. The virus was activated per manufacturer's instructions, and BHK-21 and HeLa cells were infected in serum-free medium. Transfection efficiency of recombinant virus was routinely >95% in BHK-21 cells and >50% in HeLa cells. Cells were allowed to express protein for 6 to 8 hours after infection in serum-free medium before use in experiments.
21. For cell adhesion assays and immunofluorescence, cells were suspended in basal medium (Glasco minimal essential medium, Life Technologies) containing no serum and seeded in six-well plastic plates containing coverslips coated with fibronectin (20 μg/ml). Cells attached to coverslips were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 20 min, and then incubated with antibody to Myc (9E10) at 1:300 or with antibody to β-galactosidase (β-Gal) at 1:5000 (Promega, Madison, WI) for 1 hour. Cells were then incubated for 1 hour with rhodamine phalloidin 1:500 (Sigma) or with fluorescein isothiocyanate–conjugated antibody to mouse immunoglobulin G (IgG) 1:300 (Cappel Labs, Cochranville, PA) or with both, washed with phosphate-buffered saline, and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Slides were examined with a Nikon Labophot-ZDFX-DX epifluorescence microscope, and images were photographed with a 35-mm camera and Kodak T-MAX film. For inhibition studies, various concentrations of BDM (Sigma) and ML-7 (Calbiochem, La Jolla, CA) were added to the culture medium.
22. L. P. Cramer and T. J. Mitchison, *J. Cell Biol.* **131**, 179 (1995).
23. Y. Yamakita, S. Yamashiro, F. Matsumura, *ibid.* **124**, 129 (1994).
24. L. C. Sanders and G. M. Bokoch, unpublished data.
25. M. Saitoh, T. Ishikawa, S. Matsushima, M. Naka, H. Hidaka, *J. Biol. Chem.* **262**, 7801 (1997).
26. Cell lysates from control (nontransfected) or transfected BHK-21 cells (35-mm dishes) were separated by SDS-PAGE. Equal volumes (30 μl) were separated on 6 and 15% gels, transferred to polyvinyldifluoride membrane, stained with a 2% Ponceau S solution, rinsed in water, and incubated with milk. The blot from the 6% gel was incubated with an antibody to myosin heavy chain (MHC), whereas the blot from the 15% gel was incubated with an antibody (pp2b) that only recognizes MLC phosphorylated on Ser^{19} [F. Matsumura *et al.*, *J. Cell Biol.* **140**, 119 (1998)]. Protein bands were visualized with horseradish peroxidase–conjugated goat antibody to rabbit IgG (Pierce, Rockford, IL) and with chemiluminescence.
27. M. Chrzanowska-Wodnicka and K. Burridge, *J. Cell Biol.* **133**, 1403 (1996).
28. K. Kimura *et al.*, *Science* **273**, 245 (1996).
29. M. Amano *et al.*, *J. Biol. Chem.* **271**, 29246 (1996).
30. F. Matsumura and G. M. Bokoch, unpublished data.

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Imaging Protein Kinase Cα Activation in Cells

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Spatially resolved fluorescence resonance energy transfer (FRET) measured by fluorescence lifetime imaging microscopy (FLIM), provides a method for tracing the catalytic activity of fluorescently tagged proteins inside live cell cultures and enables determination of the functional state of proteins in fixed cells and tissues. Here, a dynamic marker of protein kinase Cα (PKCα) activation is identified and exploited. Activation of PKCα is detected through the binding of fluorescently tagged phosphorylation site–specific antibodies; the consequent FRET is measured through the donor fluorophore on PKCα by FLIM. This approach enabled the imaging of PKCα activation in live and fixed cultured cells and was also applied to pathological samples.

For many proteins, there is a need to integrate spatial data with information on catalytic function. This is a particular concern in signal transduction processes, where the networking of multiple inputs affects the output, leading to grossly different cellular consequences. Methods applicable to the functional analysis of particular (signaling) proteins in situ would provide a significant advance in reaching a molecular description of cellular behavior.

The classical and novel protein kinase isotypes (cPKC and nPKC, respectively) undergo conformational changes in response to their second messenger, diacylglycerol (DAG) (*1*–*3*). This PKC activator is restricted to membrane compartments, and the stable membrane/DAG-associated complexes formed by PKC have traditionally provided a useful means of monitoring PKC isotype activation (*4*). However, PKC isotypes can associate with mem-

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Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics

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Extracellular signals regulate actin dynamics through small GTPases of the Rho/Rac/Cdc42 (p21) family. Here we show that p21-activated kinase (Pak1) phosphorylates LIM-kinase at threonine residue 508 within LIM-kinase's activation loop, and increases LIM-kinase-mediated phosphorylation of the actin-regulatory protein cofilin tenfold *in vitro*. *In vivo*, activated Rac or Cdc42 increases association of Pak1 with LIM-kinase; this association requires structural determinants in both the amino-terminal regulatory and the carboxy-terminal catalytic domains of Pak1. A catalytically inactive LIM-kinase interferes with Rac-, Cdc42- and Pak1-dependent cytoskeletal changes. A Pak1-specific inhibitor, corresponding to the Pak1 autoinhibitory domain, blocks LIM-kinase-induced cytoskeletal changes. Activated GTPases can thus regulate actin depolymerization through Pak1 and LIM-kinase.

The actin cytoskeleton is dynamic, and the rates of polymerization and depolymerization of actin are important determinants of cell motility, cell division and the formation of specialized structures. Actin polymerization drives formation and extension of lamellae at the leading edge of motile cells, while the actin-based molecular motor myosin provides the tractive force necessary for cell movement[1,2]. Members of the Rho family of GTPases regulate actin cytoskeletal dynamics by cycling between inactive GDP-bound and active GTP-bound states[3,4]. Although crosstalk among family members occurs, each is activated in response to specific environmental signals and each induces specific changes in the actin cytoskeleton: Rho induces assembly of stress fibres and focal adhesions; Rac induces peripheral actin accumulation and membrane ruffling; Cdc42 induces filopodia; and both Rac and Cdc42 assemble focal complexes[5].

Several effectors of Rho GTPases have been identified but signal-transduction pathways that link these to the actin cytoskeleton are not completely understood. A number of actin-associated proteins that regulate actin polymerization and depolymerization are potential downstream mediators. The Arp2/3 complex binds with high affinity to the pointed (more slowly growing) ends of actin filaments, and induces actin filaments to polymerize and elongate from their barbed (more quickly growing) ends[6]. WASP, the protein associated with the immune disorder Wiskott–Aldrich syndrome, and related proteins appear to be the principal regulators of Arp2/3-mediated nucleation of actin polymerization[7]. Analysis of actin polymerization *in vitro* indicates synergistic activation of actin polymerization by phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) and GTPγS-bound Cdc42 through their binding to the amino terminus of N-WASP, relieving inhibitory constraints on its carboxy terminus which can then interact with Arp2/3 and actin[8]. In addition, Rac and Rho control the severing and nucleation of new actin filaments through gelsolin, whose interaction with actin is regulated by PtdIns(4,5)P_2 (ref. 9).

Small actin-binding proteins of the cofilin/actin-depolymerizing-factor family are also important regulators of the actin cycle, mediating cleavage and disassembly of actin filaments[10]. They therefore serve as an important downstream control point for cytoskeletal dynamics[11]. LIM-kinase catalyses phosphorylation of an N-terminal serine residue of cofilin, thereby inactivating its F-actin-depolymerizing activity and leading to accumulation of actin filaments and aggregates[12,13]. LIM-kinase acts downstream of Rac but is not a direct target of this GTPase[12,13]. A protein that may link Rac and Cdc42 with LIM-kinase is the p21-activated kinase, Pak1, which, through its Rac- and Cdc42-binding domain (PBD), binds to and is activated by GTP-bound Rac and Cdc42 (refs 14, 15). Pak1 has been localized to regions of cytoskeletal assembly[16,17], and activated forms induce cytoskeletal rearrangements independently of Rac or Cdc42 activation[16–19].

Here we tested whether Pak1 could activate LIM-kinase, and used inhibitors of Pak1 and LIM-kinase *in vivo* to investigate the connections identified *in vitro*. The results define a signal-transduction pathway in which Pak1 activated by GTP-bound Rac or Cdc42 transphosphorylates and activates LIM-kinase, which in turn efficiently catalyses phosphorylation and inactivation of cofilin, resulting in decreased depolymerization of F-actin.

Results

Activation of LIM-kinase by Pak1. We measured LIM-kinase activity *in vitro* as incorporation of ^{32}P into serine residue 3 (Ser3) of cofilin[20]. Addition of a constitutively active Pak1 fused to glutathione-*S*-transferase (GST) (Pak*)[15] enhanced the rate of LIM-kinase-catalysed phosphorylation of Ser3 of cofilin 7.6-fold (Fig. 1a). Neither Pak* alone, nor a catalytically inactive LIM-kinase mutant (D460N) in the presence of Pak*, caused phosphorylation of cofilin. The N-terminal LIM and PDZ domains of LIM-kinase suppress the C-terminal kinase domain and inhibit LIM-kinase-induced cytoskeletal changes[20]. The kinase domain alone of LIM-kinase (Kd, residues 302–647) had ~2.5-fold greater activity than full-length (wild-type) LIM-kinase, and was also activated 7.6-fold by Pak* (Fig. 1a). The stimulatory effects of Pak* on LIM-kinase are thus directly due to effects on the kinase domain.

To investigate the mechanism of activation of LIM-kinase by Pak*, we tested the effects on cofilin phosphorylation of several mutations in the kinase domain of LIM-kinase. LIM-kinase contains in its activation loop a highly basic 11-amino-acid insertion (Arg495 to Arg506)[21,22] preceding a threonine (Thr508) that is analogous to a regulatory phosphorylation site in other protein

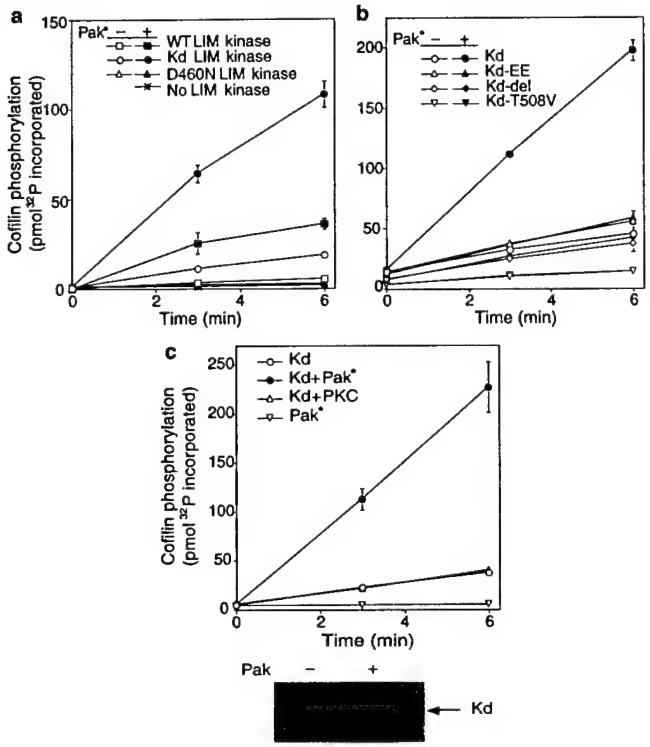

Figure 1 **Activation of LIM-kinase by Pak1 *in vitro*. a**, Increased LIM-kinase-catalysed phosphorylation of cofilin in the presence of Pak1. LIM-kinase activity was measured in the presence or absence of recombinant, constitutively active GST–Pak1 (Pak*). Equal amounts of wild-type (WT), catalytically inactive (D460N) or kinase domain only (Kd) LIM-kinase were expressed in HEK 293 cells. Cell lysates were added to reaction mixtures containing 20 μM cofilin and [γ-^{32}P]ATP, and ^{32}P incorporation into cofilin was quantified. **b**, Requirement of the activation-loop threonine and the basic insert of LIM-kinase for activation by Pak1. Kinase assays were done with equal amounts of Kd, or mutant Kd in which the basic insert was deleted (Kd-del) or in which Thr 508 was changed to a valine (T508V) or to two glutamic acid residues (-EE). Activity was determined in the presence or absence of Pak*. **c**, Phosphorylation and activation of LIM-kinase by Pak1. Kd, purified from baculovirus, was subjected to kinase assays alone or with constitutively active protein kinase C βII (PKC) or Pak*. The upper panel shows cofilin phosphorylation and the lower panel shows an autoradiogram of ^{32}P-labelled Kd resolved by SDS–PAGE.

kinases[23,24]. Mutation of Thr 508 to valine (T508V) reduced LIM-kinase activity whereas replacement with two glutamic acid residues (T508EE) resulted in an active form of LIM-kinase; neither T508V nor T508EE was activated by Pak* (Fig. 1b). Both deletion and mutation of the 11-amino-acid basic insert completely disrupted activation of LIM-kinase by Pak* (Fig. 1b). In contrast, mutation of Tyr 507 did not affect LIM-kinase activation by Pak* (data not shown). These results indicate that Pak* may activate LIM-kinase through phosphorylation of Thr 508, with the basic insert providing the substrate determinants required by Pak1 (ref. 25).

Pak*-mediated phosphorylation of the purified kinase domain (Kd) of LIM-kinase coincided with activation of this domain (Fig. 1c). Matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy identified a shift in relative molecular mass of 80,000 (from 2,098 to 2,178) in the tryptic peptide corresponding to the activation loop of LIM-kinase (^{507}YTVVGNPYWMAPGMINGR) after phosphorylation of purified Kd by Pak1. This confirmed that Thr 508 was the site phosphorylated by Pak*. Pak* also phosphorylated a catalytically inactive form of Kd (Kd(D460N)), but not Kd(T508EE) (data not shown). These results, coupled with the low activity of the T508V mutant and loss of LIM-kinase activity upon phosphatase treatment[20], indicate that phosphorylation of Thr 508 is required for maximal LIM-kinase activity. Protein kinase C βII, protein kinase A and phosphoinositide-dependent protein kinase-1 were unable to phosphorylate or activate LIM-kinase.

Binding of activated Rac or Cdc42 to the N-terminal PBD in Pak1 induces a conformational change that relieves inhibitory constraints on the C-terminal kinase domain[14,15,26]. The active Pak1 kinase domain (amino acids 232–504, Pak-KD(Kin$^+$)) enhanced LIM-kinase activity roughly twofold (Fig. 2A, a). Neither the catalytically inactive kinase domain (Pak-KD(Kin$^-$) (Fig. 2A, a) nor the full-length catalytically inactive GST–Pak1(K299R) fusion protein (Fig. 2A, b) activated LIM-kinase. Although an N-terminal PBD-containing fragment of Pak1 (Pak1 amino acids 67–150) alone had no effect, the full-length Pak* activated LIM-kinase more effectively than Pak-KD(Kin$^+$) (Fig. 2A, a), indicating that the presence of an intact Pak1 N terminus enhances LIM-kinase activation.

Histidine-tagged full-length Pak1 (wild-type Pak1) is not constitutively active and remains GTPase dependent for full activity[27]. Wild-type Pak1 alone did not activate LIM-kinase (Fig. 2B). Addition of inactive GDP-bound Cdc42 to wild-type Pak1 activated LIM-kinase ~2-fold whereas active GTPγS-bound Cdc42 activated LIM-kinase ~11-fold, increasing LIM-kinase activity from 5.5±0.4 to 64±0.9 mol phosphate incorporated per mol enzyme per minute. This is comparable to activities reported for other purified serine/threonine kinases[28]. GTPγS-bound Rac similarly induced Pak1 activation of LIM-kinase activity (data not shown). Thus the GTPases Rac and Cdc42 may be connected with the actin cytoskeleton through the effectors Pak1 and LIM-kinase.

Rac/Cdc42-dependent interaction of Pak1 with LIM-kinase. We used co-immunoprecipitation to investigate *in vivo* interactions between LIM-kinase and Pak1. We immunoprecipitated Pak1 from A431 cells, and probed immunoprecipitates with a chicken antibody directed to an internal peptide in LIM-kinase. The two proteins are associated *in vivo* (Fig. 3a). To identify factors that regulate the association between Pak and LIM-kinase, we used co-transfections in 293 cells. Constitutively activated Rac(Q61L) enhanced complex formation between LIM-kinase and Pak, compared with the complex formation induced by inactive Rac(T17N) (Fig. 3b, lanes 1, 2). The partially activated Pak1(H83,86L) mutant, which is defective in Rac and Cdc42 binding[18], also bound LIM-kinase (Fig. 3b, lane 3). Pak1 and the kinase domain of LIM-kinase also formed complexes, consistent with the ability of Pak1 to catalyse phosphorylation and induce activation of Kd.

We mapped the sites of interaction between LIM-kinase and Pak1 by binding of ^{35}S-labelled LIM-kinase to Pak1 fragments expressed as GST fusion proteins (Fig. 3c). LIM-kinase bound specifically to both the N-terminal regulatory and the C-terminal catalytic domains of Pak1. We localized the LIM-kinase-binding region in the Pak1 N terminus to the region between amino acids 67 and 150, which contains the p21-binding domain (amino acids 67–86) and the autoinhibitory regulatory domain (amino acids 83–149), indicating that binding of Pak 1 to activated Rac or Cdc42 would regulate binding of LIM-kinase to Pak1. Addition of activated Cdc42 to wild-type Pak1 or to the Pak1 PBD (amino acids 67–150) enhanced binding to LIM-kinase, but did not affect the interaction of LIM-kinase with the non-p21-binding mutant Pak1(H83,86L) or with the Pak1 C-terminal kinase domain (Fig. 3c). In a converse experiment, Cdc42–GTPγS enhanced binding of soluble wild-type Pak1 to GST–LIM-kinase (data not shown). Thus LIM-kinase binds to a region of Pak1 that includes its autoinhibitory domain, and this interaction is increased by Rac or Cdc42 binding to the PBD.

***In vivo* effects on the actin cytoskeleton.** To assess the role of LIM-kinase as a downstream mediator of Rac and Cdc42 in actin cytoskeletal reorganization, we co-transfected BHK cells with wild-type LIM-kinase or a catalytically inactive mutant (D460N) together with constitutively active Rac(Q61L) or Cdc42(Q61L). Although both Rac and Cdc42 induced a similar peripheral reor-

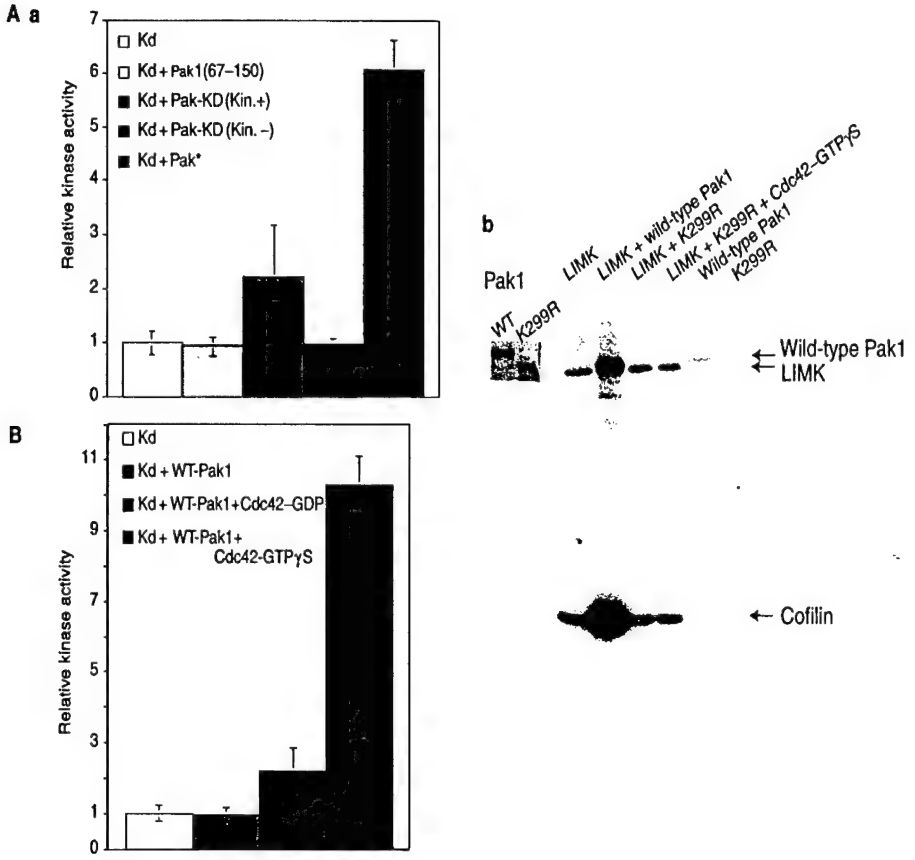

Figure 2 **Requirement of Pak1 activation and kinase activity for regulation of LIM-kinase. A**, Requirement for intrinsic kinase activity and an intact N terminus of Pak1 for activation of LIM-kinase. **a**, Kd-catalysed phosphorylation of cofilin was measured in the presence or absence of GST fusion proteins containing the N-terminal regulatory domain of Pak1 (Pak(67–150)), the active kinase domain of Pak1 (Pak-KD(Kin.$^+$)), the catalytically inactive kinase-domain mutant Pak1(K299R) (Pak-KD(Kin.$^-$)) or Pak*. Relative kinase activity was calculated from linear rates of cofilin phosphorylation and the basal activity of Kd was set to 1. **b**, Kinase assays were carried out using wild-type LIM-kinase (LIMK) and cofilin in the presence or absence of a constitutively active Pak1 fused to GST (wild-type Pak1) or a catalytically inactive Pak1 mutant, K299R. Reaction products were separated by SDS–PAGE and detected by autoradiography. In addition to Pak1-catalysed phosphorylation of LIM-kinase, both LIM-kinase and Pak1 autophosphorylate. Inset at left, Coomassie blue-stained gel of the wild-type (WT) and K299R Pak1–GST fusion proteins used in the kinase assays. **B**, Maximal stimulation of LIM-kinase by Cdc42–GTPγS-activated Pak1. Purified Kd was subjected to kinase assays alone or in combination with histidine-tagged Pak1 (WT-Pak1), without or with inactive GDP-bound (Cdc42–GDP) or active GTPγS-bound (Cdc42–GTPγS) Cdc42, as described in the Methods.

ganization and thickening of tangential actin fibres, Rac also induced prominent membrane ruffles whereas Cdc42 induced microspikes (Fig. 4). Expression of wild-type LIM-kinase increased the number and size of peripheral actin fibres. Expression of LIM-kinase(D460N) disrupted Rac- and Cdc42-induced peripheral actin reorganization and Rac-induced membrane ruffling, but did not affect Cdc42-induced microspike formation. Quantitatively, peripheral reorganization and thickening of actin fibres was observed in ~50% of the cells expressing Cdc42(Q61L), in ~80% of cells expressing Cdc42(Q61L) plus wild-type LIM-kinase, and in only 5% of cells expressing Cdc42(Q61L) plus LIM-kinase(D460N). LIM-kinase(D460N) did not significantly reduce Cdc42-induced microspikes in these cells. Like the D460N mutant, catalytically inactive LIM-kinase(T508V) also inhibited Rac-induced actin reorganization (data not shown). Together, these data indicate that LIM-kinase may be downstream of Rac-induced cytoskeletal signalling, but mediates only a subset of Cdc42-induced cytoskeletal changes.

To determine whether the LIM-kinase-induced cytoskeletal changes depended on Pak1 activity, we transfected BHK cells with LIM-kinase and the N-terminal autoregulatory domain of Pak1 (Pak(83–149)), which inhibits Pak activity *in vivo*[26,29]. Although control and Pak(83–149)-expressing BHK cells had a very organized actin cytoskeleton, composed of parallel actin fibres, cells expressing LIM-kinase had an exaggerated membrane ruffling phenotype and accumulated large aggregates of actin (Fig. 5, compare a, c). These changes induced by LIM-kinase presumably reflect severe inhibition of F-actin depolymerization as a result of inactivation of cofilin[12,13,20]. Co-expression of Pak(83–149) with LIM-kinase resulted in substantial inhibition of LIM-kinase-induced cytoskeletal changes (Fig. 5d). Changing Leu 107 to phenylalanine, which inactivates Pak1's autoinhibitory domain[26], abolished the ability of the Pak(83–149) autoinhibitor to block LIM-kinase-induced cytoskeletal changes (Fig. 5, compare d, e). The distribution of actin cytoskeletal phenotypic changes was scored to provide an assessment of the effects of Pak(83–149) on LIM-kinase-induced changes[20]. When wild-type LIM-kinase and the inactive Pak1 inhibitor Pak1(L107F) were coexpressed, ~80% of the cells exhibited moderate to severe changes in the actin cytoskeleton, consisting of loss of stress fibres and increasing accumulation of aggregates of actin (2+ to 4+, as described in ref. 20). In contrast, when wild-type LIM-kinase and Pak(83–149) were coexpressed, ~80% of the cells exhibited no or minimal actin cytoskeletal changes (0 to 1+). These data indicate that LIM-kinase activity is dependent on Pak1 activity *in vivo*.

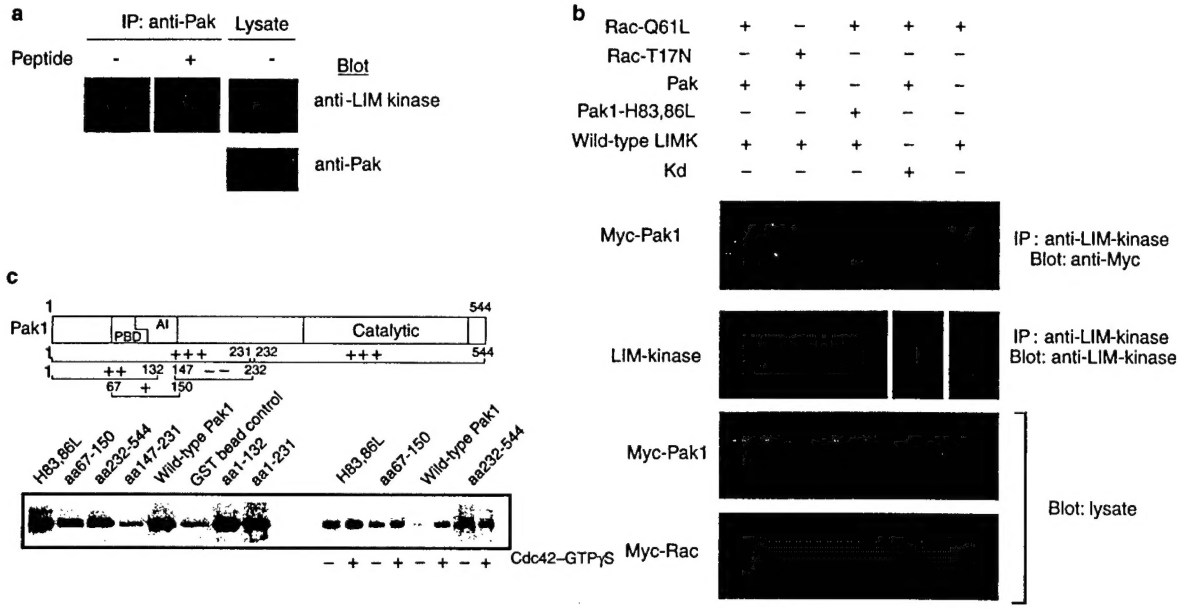

Figure 3 **Direct interaction of LIM-kinase and Pak1. a**, Association of endogenous Pak1 with LIM-kinase. Pak1 was immunoprecipitated (IP) with rabbit polyclonal anti-Pak1 antibody and western blots of immunoprecipitates were probed with chicken anti-LIM-kinase antibody 625 (ref. 20) in the absence or presence of the immunizing peptide (residues 255–271 of human LIM-kinase). The peptide reduced the amount of LIM-kinase detected in Pak1 immunoprecipitates by 50–60%. **b**, Increased binding of Pak1 to LIM-kinase in cells expressing activated Rac. The indicated combinations were transfected into HEK293 cells. The cells were lysed after 48 h and LIM-kinase was immunoprecipitated with the 5079 antibody. The immunoprecipitates were blotted and probed for co-immunoprecipitated Myc-tagged Pak1 with the 9E10 antibody against the Myc tag (top panel).The blot was stripped and reprobed with the chicken anti-LIM-kinase antibody 625 to compare LIM-kinase expression in immunoprecipitates (second panel), and expression of Myc–Pak and Myc–Rac was verified by probing lysates with the 9E10 antibody (bottom two panels). The experiment was repeated four times with similar results. **c**, Binding of LIM-kinase to regions in the N and C termini of Pak1. Top, the amino-acid residues present in each fragment of human Pak1 are indicated. The square brackets indicate the Pak1 fragments, and plus or minus symbols indicate qualitatively the relative amount of binding of each fragment to LIM-kinase. Bottom, the indicated GST–Pak1 fragments and mutants were tested for binding to [^{35}S]methionine-labelled full-length LIM-kinase *in vitro*. We also tested a non-GTPase-binding full-length Pak1 (H83,86L)[18] and full-length wild-type Pak1. GST-bound beads and beads to which GST–Nck was bound (data not shown) did not specifically bind LIM-kinase. The right panel shows results obtained from use of GST fusions containing Pak1(H83,86L), an N-terminal fragment of Pak1 (amino acids 67–150) containing the p21-binding domain (PBD) and autoinhibitory domain (AI), wild-type Pak1 and the C-terminal catalytic domain (amino acids 232–544) to determine the effects of Cdc42–GTPγS-induced activation of Pak1 on Pak1's ability to bind LIM-kinase *in vitro*. GST fusions containing wild-type Pak1 bound 5% of the input *in vitro*-translated [^{35}S]LIM-kinase whereas GST, GST–Nck or GST–Pak(147–231) bound <0.5% of the input counts. The experiments were repeated with internal controls three times, with similar results. aa, amino acids.

Changing Thr 508 to valine or removal of the basic insert in the kinase domain of LIM-kinase abrogates its activity *in vivo*[20]. Deletion of the basic insert abolished activation by Pak1 but did not abolish basal kinase activity *in vitro* (Fig. 1b). These results indicate a strict requirement for phosphorylation of Thr 508 for *in vivo* activity of LIM-kinase. Blocking LIM-kinase-induced cytoskeletal changes with the intact, but not the mutant, Pak1 autoinhibitor supports the conclusion that Pak1 activity is essential for LIM-kinase activity *in vivo*.

Inhibition of Rac-and Cdc42-induced cytoskeletal changes by catalytically inactive LIM-kinase(D460N), coupled with inhibition of LIM-kinase-induced cytoskeletal changes by the Pak1 inhibitor, support the idea that information is transferred from Rac to Pak to LIM-kinase. If this is correct, then dominant-negative LIM-kinase should interfere with Pak1-induced cytoskeletal changes. The Pak1(H83,86L) mutant is constitutively active and induces cytoskeletal changes, including dorsal membrane ruffles, formation of lamellipodia, small actin aggregates, cell polarization and focal-complex formation[17–19]. In BHK cells, the prominent phenotypes induced by Pak1(H83,86L) include large dorsal ruffles, thickening of actin cables and actin aggregation (Fig. 6a, b). Similar formation of dorsal ruffles that contain Pak1 has been shown to occur on treatment of cells with platelet-derived growth factor and insulin[17]. Addition of wild-type LIM-kinase increased actin aggregation in cells expressing Pak1(H83,86L) (Fig. 6c). However, coexpression of dominant-negative LIM-kinase and Pak1(H83,86L) blocked both formation of the large dorsal ruffles and the actin aggregation induced by Pak1(H83,86L) (Fig. 6d). Because the level of expression of transfected proteins varies within cells of a population, we scored the formation of dorsal ruffles seen with Pak1(H83,86L) and the actin aggregation characteristic of LIM-kinase activity in expressor cells to provide an estimate of intrinsic activity in the population[12,20]. The dominant-negative LIM-kinase(D460N) abolished Pak1(H83,86L)-induced dorsal ruffles and decreased by 50% the number of cells exhibiting actin aggregates (Fig. 6g). As previous studies indicated that dominant-negative LIM-kinase blocked the actin cytoskeletal effects induced by wild-type LIM-kinase[12,13,20], these results indicate that LIM-kinase mediates some of the cytoskeletal effects of Pak1.

Because Pak1 has been reported to have kinase-independent effects on the cytoskeleton[18,19], we used a catalytically inactive Pak (H83,86L,K299R) to test whether dominant-negative LIM-kinase(D460N) could block these effects. Pak1(H83,86L,K299R) caused membrane extensions, but not dorsal or peripheral ruffles, in BHK cells (Fig. 6e). These membrane extensions were not blocked by LIM-kinase(D460N) (Fig. 6f). Of cells expressing Pak1 (H83,86L,K299R), ~50% exhibited some membrane extensions. This fraction did not change in cells coexpressing both the kinase-inactive Pak1 and the kinase-inactive LIM-kinase. Thus the kinase-independent effects of Pak1 are not dependent on LIM-kinase.

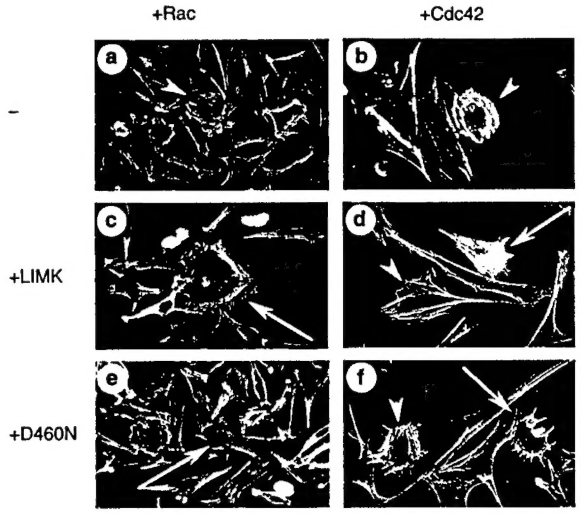

Figure 4 **Inhibition of Rac- and Cdc42-induced cytoskeletal changes by dominant-negative LIM-kinase.** BHK cells were stained for actin with fluorescently labelled phalloidin, and cells expressing Rac (**a**, **c**, **e**), Cdc42 (**b**, **d**, **f**) and wild-type (LIMK; **c**, **d**) or D460N (**e**, **f**) LIM-kinase constructs were identified by immunostaining as described in Methods. Cells expressing Rac or Cdc42 only are indicated by an arrowhead; arrows show cells expressing both the GTPase and LIM-kinase. 50–100 cells from three independent transfections were scored for each panel.

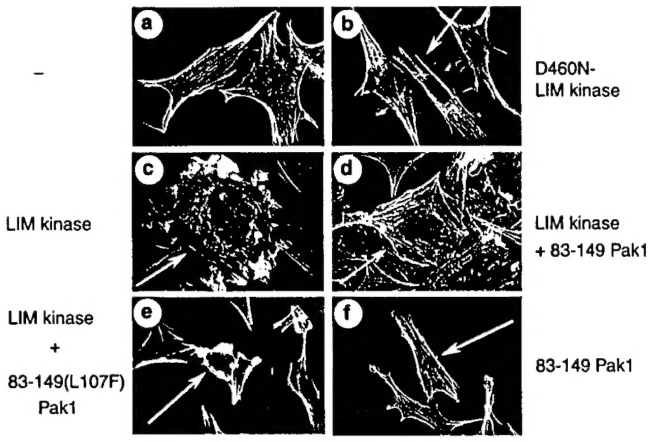

Figure 5 **Inhibition of LIM-kinase-induced actin cytoskeletal changes by the autoinhibitory domain of Pak1.** BHK cells were transfected with **b**, D460N mutant LIM-kinase; **c**, wild-type LIM-kinase; **d**, wild-type LIM-kinase plus the Pak1 autoinhibitory domain (residues 83–149); **e**, wild-type LIM-kinase plus an inactive Pak1 autoinhibitory domain (83–149(L107F)); or **f**, the Pak1 autoinhibitory domain alone, as described in Methods. **a**, Untransfected cells. Arrows show cells expressing the indicated protein(s). 50–100 transfected cells were scored for actin cytoskeletal changes as described[20].

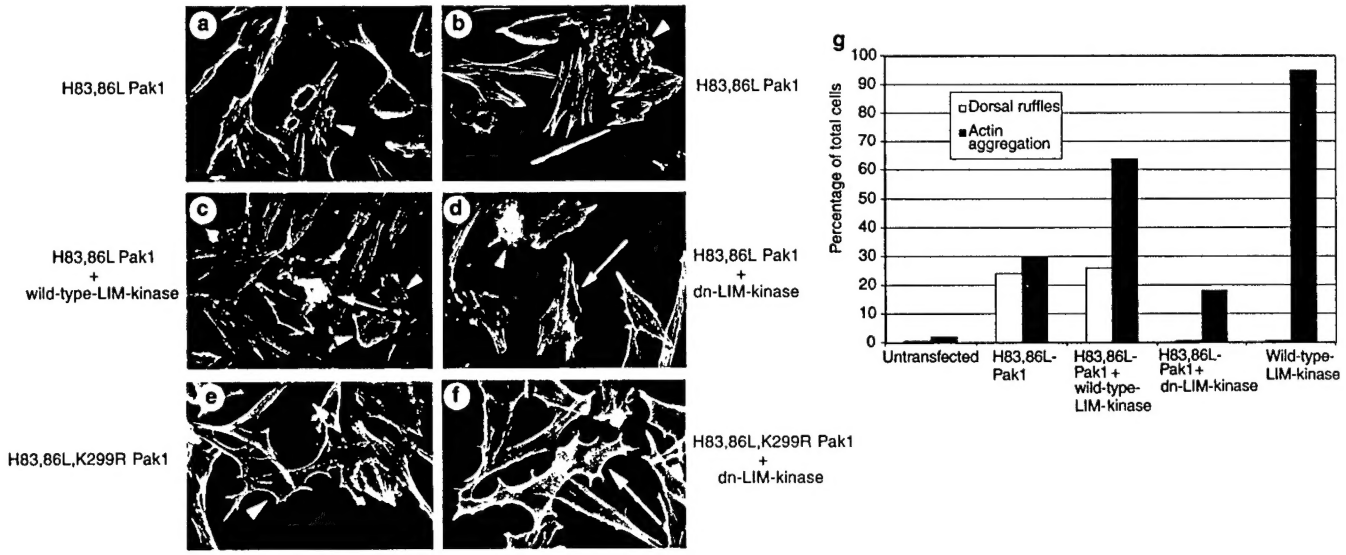

Figure 6 **Inhibition of Pak1(H83,86L)-induced cytoskeletal changes by dominant-negative LIM-kinase(D460N).** BHK cells expressing Pak1(H83,86L) or catalytically inactive Pak1(H83,86L,K299R) together with wild-type or dominant negative (D460N; dn) LIM-kinase were identified by double immunofluorescence as described in Methods. Phalloidin-stained actin cytoskeletal changes are shown in cells expressing Pak1(H83,86L) and LIM-kinase. Arrowheads show cells expressing Pak1(H83,86L) alone and arrows show cells expressing Pak1(H83,86L) plus wild-type or dn-LIM-kinase. **a**, **b**, Large dorsal ruffles, thickening of peripheral actin cables and aggregates of actin are present in cells expressing Pak1(H83,86L) alone. **c**, LIM-kinase enhances actin aggregation. **d**, Inhibition of dorsal ruffles and actin aggregation by coexpression of dn-LIM-kinase with Pak1(H83,86L). **e**, Catalytically inactive Pak1(H83,86L,K299R) induced membrane extensions but no large dorsal ruffles or actin aggregates. **f**, Coexpression of dn-LIM-kinase did not block the effects of catalytically inactive Pak1(H83,86L,K299R). **g**, Distribution of actin cytoskeletal phenotypes in the population of cells expressing the indicated proteins. 50–100 cells per transfection were scored in a single-blind manner by two independent observers. Each transfection was repeated three times with similar results.

Upon coexpression of Pak1 and LIM-kinase, both proteins localized with actin in the resulting exaggerated membrane ruffles (Fig. 7). The two proteins also co-localized with actin aggregates (data not shown). Coexpression of LIM-kinase with green fluorescent protein did not result in co-localization at membrane ruffles or actin aggregates (data not shown), indicating that the *in vivo* association of LIM-kinase and Pak is specific. These findings, together with the protein-interaction data shown in Fig. 3, indicate that a Pak1–LIM-kinase complex may be localized to the area at which actin cytoskeleton rearrangements occur.

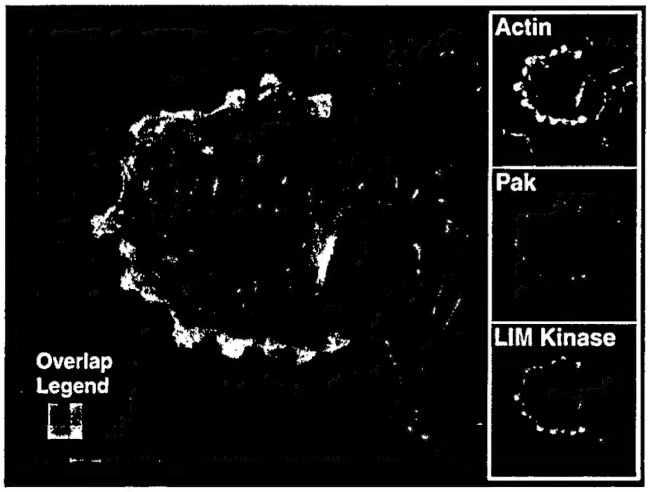

Figure 7 **Co-localization of Pak and LIM-kinase with actin at membrane ruffles.** Cos-7 cells were transfected with wild-type LIM-kinase and Myc-tagged wild-type Pak1 (Pak). The cells were then stained for LIM-kinase (rhodamine), Pak1 (AMCA-S) and F-actin (phalloidin) as described in Methods. The staining legend in the bottom left corner indicates the composite colours resulting from the overlap of any two or all three fluorescent markers.

Discussion

Our results indicate that the cofilin-phosphorylating protein LIM-kinase is activated by the Rac- and Cdc42-dependent protein kinase Pak1. Maximal activation by Pak1 requires its intrinsic kinase activity and regions in its N and C termini that may represent one constitutive and one regulated binding site. Activation of LIM-kinase depends on phosphorylation of Thr 508 in the activation loop of its kinase domain by Pak1. Mutational inactivation of the second LIM domain or of the PDZ domain, or deletion of the N-terminal region containing both the LIM and the PDZ domains, increased LIM-kinase activity *in vivo*[20]. Moreover, the N terminus inhibited activity of the kinase domain of LIM-kinase. Relief of inhibitory constraints imposed by the N terminus is thus necessary for full activation of LIM-kinase, implying that regulatory mechanisms in addition to Pak1 control LIM-kinase activity.

In agreement with the findings of refs 12, 13, LIM-kinase appears to mediate actin cytoskeletal responses to Rac. The time course of insulin-induced activation of LIM-kinase is reported to parallel that of insulin-induced membrane ruffling, a process dependent on Rac[13]. The time course of insulin-induced activation of Pak1 is similar[30]. These data are consistent with ligand-dependent regulation of cytoskeletal dynamics through a Rac→Pak→LIM-kinase pathway but, because responses are localized within the cell, techniques to assess local changes in activity will need to be developed to order physiological responses precisely. Moreover, although GTP-bound Rac and Cdc42 both activate Pak1, catalytically inactive LIM-kinase blocked only a part of the morphological response to Cdc42: Cdc42-induced microspikes persisted, consistent with the idea that distinct signalling pathways mediate the specific morphological responses to each of the Rho-family GTPases[5]. Our results confirm those of ref. 12 but also indicate that only a subset of the cytoskeletal responses to Cdc42 is mediated by LIM-kinase. Cdc42's effects on microspike formation may be mediated by distinct pathways including effects of N-WASP on nucleation of actin polymerization[31].

Pak1 has dramatic effects on cytoskeletal dynamics and Pak1 function is required for both fibroblast[32] and endothelial-cell motility (W.B. Kiosees, R.H. Daniels, C. Otey, G.M. Bokoch and M.A. Schwartz, unpublished observations), as well as for formation of neurites[19]. There is evidence that Pak1 can regulate the actin–myosin cytoskeleton both through N-terminal-interacting proteins and by phosphorylating specific substrates. For example, it can modulate cell contractility through phosphorylation and inhibition of myosin-light-chain kinase[33]. Previous work[18] showed that, upon transient overexpression in fibroblasts, Pak1(H83,86L) induced cell polarization accompanied by peripheral and dorsal membrane ruffling. When Pak1 catalytic function was inactivated, cells failed to polarize, and peripheral, but never dorsal, ruffling was seen. These results led to the conclusion that Pak1 could induce ruffling in the absence of kinase activity. More recent work[32] using cell lines in which Pak1 expression could be carefully controlled established that Pak1(H83,86L) induced polarized ruffles associated with directional cell movement. In contrast, the catalytically inactive version induced random ruffling and the cells were unable to undergo directed migration. Interestingly, in these same studies, low-level expression of catalytically activated Pak1(T423E) also induced a polarized ruffling phenotype. Our results, obtained using BHK cells, support these findings. We observed that, although expression of Pak1(H83,86L) induced extensive dorsal ruffling and actin thickening, this phenotype was almost totally absent in the presence of the kinase-inactive version of the Pak1(H83,86L) mutant. Instead, this mutant caused the formation of membrane extensions that were insensitive to the expression of a dominant-negative form of LIM-kinase.

Our present view is that Pak1 can induce ruffling by at least two mechanisms: a preferred, physiologically relevant pathway that requires the kinase function and which is important for directed cell motility, and a second mechanism that is revealed by the catalytically inactive Pak1. Our data establish that LIM-kinase is an important mediator of the preferred pathway. LIM-kinase acts to promote and stabilize actin structures formed in response to Pak1 or other cytoskeletal effectors of Rac and Cdc42.

Our results thus define a signal-transduction pathway through which GTP-bound Rac (and Cdc42) activate Pak1 to allow it to form a complex with, and phosphorylate, LIM-kinase. Activated LIM-kinase catalyses phosphorylation and consequent inactivation of cofilin. Inhibition of actin depolymerization represents an essential control point in the overall cycle of actin polymerization/depolymerization, resulting in cytoskeletal changes[11]. One important pathway for transmission of extracellular signals to the actin cytoskeleton is through the Rac/Cdc42→Pak1→LIM-kinase→cofilin cascade. Signalling through the WASP family of proteins, which primarily affect actin polymerization[7,8], must be coordinated with effects on actin depolymerization to provide the high degree of resolution of cell responses observed in response to extracellular signals. For example, responses to Cdc42 would probably induce actin cytoskeletal changes by coordinately stimulating nucleation of actin polymerization through N-WASP and Arp2/3, and blocking depolymerization through Pak1- and LIM-kinase-mediated inactivation of cofilin. □

Methods

Preparation of LIM-kinase and Pak proteins.

Cell lysates of transiently transfected HEK293 cells expressing either wild-type, catalytically inactive dominant-negative (D460N), phosphorylation-site mutant (T508V), catalytically active phosphorylation-site mutant (-EE), or basic-insert-deletion mutant (-del) LIM-kinase-1 (ref. 20) were prepared 60–72 h post-transfection, lysed in PBS with 0.25% Triton-X100, mixed with glycerol to 25%, and frozen at –80 °C. The purified kinase domain of LIM-kinase (Kd) was prepared by cloning complementary DNA encoding the kinase domain (amino acids 302–647) of human LIM-kinase-1 into the baculovirus expression vector pAcGHLT (Pharmingen). The detergent soluble supernatant from infected SF21 cells was bound to glutathione–agarose beads and digested with thrombin for 1.5h according to the manufacturer's protocols (Pharmingen). Protein was combined with glycerol to 25%, and stored at –80 °C. All Pak1 constructs were expressed and purified as described[13,24].

Kinase assays.

In vitro kinase reactions were run as described[20] using 200 μM [γ-^{32}P]ATP, and ^{32}P incorporation into trichloroacetic acid insoluble protein was measured. Kinase activity was adjusted on the basis of protein expression level, and quantified by chemiluminescence imaging using Molecular Dynamics (BioRad) hardware and software. Phosphorylated cofilin accounted for >95% of the incorporated radioactivity. All assays were done in triplicate and repeated at least six times with similar results. Kinase assays for Fig. 1a, b were done using detergent-soluble (0.25% Triton-X100) HEK293 cell lysates 72 h after transfection. All

other kinase assays were done with the purified kinase domain (Kd) of LIM-kinase. Purified Kd was combined with stoichiometrically equivalent amounts of wild-type Pak1 and either Cdc42 or Rac that had been loaded and activated with the non-hydrolysable GTP analogue GTP-γS as described[15]. A mutant cofilin, with the Ser 3 residue replaced by an alanine, was not phosphorylated by LIM-kinase[20]. Kinase reactions for SDS–PAGE analysis were stopped by addition of Laemmli sample buffer and samples visualized by autoradiography after SDS–PAGE on 13% gels.

MALDI mass spectrophotometry.

Mass-spectrophotometry analysis of phosphorylated LIM-kinase was adapted from ref. 34. GST–Kd expressed from baculovirus was phosphorylated in kinase reaction buffer with a substoichiometric amount of Pak* and digested with 1.5 μg ml^{-1} modified trypsin (Promega) The solubilized, digested proteolytic fragments were loaded on a MALDI mass spectrophotometer and analysed for 80K shifts in known proteolytic fragments.

Co-immunoprecipitation of Pak1 and LIM-kinase.

HEK292 cells transfected with LIM-kinase, Myc–Pak1 and Myc–Rac were prepared as described for kinase reactions. The detergent-soluble fraction was incubated with rabbit anti-LIM-kinase antibody 5079 (ref. 20) and protein-A–agarose for 1.5 h, washed, separated by SDS–PAGE and immunoblotted with mouse anti-Myc monoclonal antibody 9E10 to detect Pak1. The blot was stripped and LIM-kinase in immunopreciptates was detected with the chicken anti-LIM-kinase antibody 625 (ref. 20). Expressed Myc–Pak and Myc–Rac were identified by immunoblotting soluble lysates, and levels of protein expression were determined to be roughly equal under each condition.

^{35}S-labelling and GST pulldowns of LIM-kinase.

LIM-kinase was labelled with ^{35}S using the Promega TNT T7 Quick Coupled transcription/translation system in the presence of 1 mM $ZnCl_2$ and [^{35}S]methionine. GST–Pak1 fusion proteins were prepared as described[15,34]. For interaction assays, [^{35}S]LIM-kinase was incubated with equal amounts of various forms of GST–Pak1 bound to glutathione beads for 30 min on ice in a 250 μl volume of 10 mM PIPES, pH 7.3, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, and then washed extensively. Where indicated, Cdc42 preloaded with GTP-γS was included. Bound proteins were visualized by autoradiography after SDS–PAGE on 13% gels.

Immunocytochemistry.

For BHK-cell immunofluorescence, cells were plated, grown and transfected on glass coverslips. For Cos-7-cell immunofluorescence, cells were plated and transfected as described[20]. Pak1, Rac and Cdc42 expression was detected using an anti-Myc monoclonal antibody (9E10; 1:1,000 dilution), while LIM-kinase was detected using the LIM-kinase-specific antibody 5079 (1:1,250). Actin was detected using an Oregon green (fluorescein)-tagged phalloidin (1:200), Myc-tagged Pak, Rac and Cdc42 with AMCA-S-conjugated anti-mouse IgG (1:200), and LIM-kinase with Texas red (rhodamine)-conjugated anti-rabbit IgG (1:200) (Molecular Probes, Eugene, OR). Cells were equilibrated and mounted with Pro-Long mounting media. Micrographs were taken with a Zeiss Axiophot microscope with an attached Hamamatsu colour, chilled, charge-coupled-device camera using ×40 and ×60 objectives.

Pak and LIM-kinase constructs expressed in BHK and HEK 293 cells were in the expression vector pcDNA-3 (Invitrogen). LIM-kinase expression vectors were transfected into BHK cells using Superfect (Qiagen) or Gene PORTER (GTS, La Jolla, CA) 30 h before fixation. All Pak1, Rac and Cdc42 Myc-tagged constructs expressed in BHK cells were transfected using the Semliki Forest virus (SFV) gene expression system (Life Technologies). Virus was prepared and activated according to the manufacturer's instructions, and BHK-21 cells were infected in serum-free media. Cells were allowed to express protein for 8–18 h before experiments.

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1. Condeelis, J. Life at the leading edge: the formation of cell protrusions. *Annu. Rev. Cell Biol.* **9**, 411–444 (1993).
2. Mitchison, T. J. & Cramer, L. P. Actin-based cell motility and cell locomotion. *Cell* **84**, 371–379 (1996).
3. Hall, A. Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514 (1998).
4. Ridley, A. J. Rho: theme and variations. *Curr. Biol.* **6**, 1256–1264 (1996).
5. Mackay, D. J. G. & Hall, A. Rho GTPases. *J. Biol. Chem.* 273, 20685–20688 (1998).
6. Mullins, R. D., Heuser, J. A. & Pollard, T. D. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl Acad. Sci USA* 95, 6181–6186 (1998).
7. Machesky, L. M. & Insall, R. H. Scar 1 and the related Wiscott-Aldrich syndrome protein, WASP regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* **8**, 1347–1356 (1998).
8. Rohatgi, R. *et al.* The interaction between N-WASP and the Arp2/3 complex links Cdc 42-dependent signals to actin assembly. *Cell* 97, 221–231 (1999).
9. Hartwig, J. H. *et al.* Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* **82**, 643–653 (1995).
10. Moon, A. L. & Drubin, D. G. The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics. *Mol. Biol. Cell* **6**, 1423–1431 (1995).
11. Theriot, J. A. Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton. *J. Cell Biol.* **136**, 1165–1168 (1997).
12. Arber, S. *et al.* Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 393, 805–809 (1998).
13. Yang, N. *et al.* Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 393, 809–812 (1998).
14. Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S., & Lim, L. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 367, 41–46 (1994).
15. Knaus, U. G., Morris, S., Dong, H. J., Chernoff, J.& Bokoch, G. M. Regulation of human leukocyte p21-activated kinases through G protein-coupled receptors. *Science* 269, 221–223 (1995).
16. Manser, E. *et al.* Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Mol. Cell Biol.* **17**, 1129–1143 (1997).
17. Dharmawardhane, S., Sanders, L. C., Martin, S. S., Daniels, R. H. & Bokoch, G. M. Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. *J. Cell Biol.* **138**, 1265–1278 (1997).
18. Sells, M. A. *et al.* Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr. Biol.* **7**, 202–210 (1997).
19. Daniels, R. H., Hall, P. S. & Bokoch, G. M. Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells. *EMBO J.* **17**, 754–764 (1998).
20. Edwards D. C. & Gill, G. N. Structural features of LIM kinase that control effects on the actin cytoskeleton. *J. Biol. Chem.* **274**, 11352–11361 (1999).
21. Bernard, O., Ganiatsa, S., Kannourakis, G. & Dringen, R. Kiz-1, a protein with LIM zinc finger and kinase domains, is expressed mainly in neurons. *Cell Growth Differ.* **5**, 1159–1171 (1994).
22. Mizuno, K. *et al.* Identification of a human cDNA encoding a novel protein kinase with two repeats of the LIM/double zinc finger motif. *Oncogene* **9**, 1605–1612 (1994).
23. Adams, J. A., McGlone, M., Gibson, R. & Taylor, S. S. Phosphorylation modulates catalytic function and regulation in the cAMP-dependent protein kinase. *Biochemistry* **34**, 2447–2454 (1995).
24. Johnson, L. N. & O'Reilly, M. Control by phosphorylation. *Curr. Opin. Struct. Biol.* **6**, 762–769 (1996).
25. Tuazon, P. T., Spanos, W. C., Gump, E. L., Monnig, C. A. & Traugh, J. A. Determinants for substrate phosphorylation by p21-activated protein kinase (gamma-PAK). *Biochemistry* **36**, 16059–16064 (1997).
26. Zenke, F. T., King, C. C., Bohl, B. P. & Bokoch, G. M. Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. *J. Biol. Chem.* (in the press).
27. Bokoch, G. M. *et al.* A GTPase-independent mechanism of p21-activated kinase activation. *J. Biol. Chem.* 273, 8137–8144 (1998).
28. Bossemeyer, D., Kinzel, V & Reed, J. in *Protein Phosphorylation* (ed. Marks, F.) 37–74 (VCH, Weinhiem, Germany, 1996).
29. Zhao, Z. S. *et al.* A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol. Cell Biol.* **18**, 2153–2163 (1998).
30. Tsakiridis, T., Taha, C., Grinstein, S. & Klip, A. Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. *J. Biol. Chem.* **271**, 19664–19667 (1996).
31. Miki, H., Sasaki, T., Takai, Y. & Takenawa, T. Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. *Nature* 391, 93–96 (1998).
32. Sells, M. A., Boyd, J. T. & Chernoff, J. p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. *J. Cell Biol.* **145**, 837–849 (1999).
33. Sanders, L. C., Matsumura, F., Bokoch, G. M. & de Lanerolle, P. Inhibition of myosin light chain kinase by p21-activated kinase. *Science* **283**, 2083–2085 (1999).
34. Helmann, U., Wernstedt, C., Gonez, J. & Heldin, C. Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochm.* **224**, 451–455 (1995).

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